

# Pro-urokinase and prekallikrein are both associated with platelets

## Implications for the intrinsic pathway of fibrinolysis and for therapeutic thrombolysis

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The contact-dependent intrinsic pathway of fibrinolysis involving factor XII, prekallikrein (PK) and pro-urokinase (pro-UK) remains poorly understood. Casein autography of washed, intact platelets revealed both PK and pro-UK. Accordingly, platelets may mediate physiological thrombolysis by this pathway since factor XIIa activates PK and kallikrein activates pro-UK. Acid washing dissociated PK but not pro-UK from platelets. Exogenous pro-UK was specifically incorporated by platelets from the ambient fluid and similarly could not be dissociated from intact platelets. Therefore, platelets may also mediate an effect from therapeutically administered pro-UK by prolonging its half-life.

Pro-urokinase; Prekallikrein; Platelet; Fibrinolysis

### 1. INTRODUCTION

Pro-urokinase (pro-UK) is a plasminogen proactivator which is activated to urokinase (UK) by both plasmin [1] and kallikrein [2]. Factor XIIa activates plasma prekallikrein (PK) [3], and initiates the contact pathway of blood coagulation which has also long been implicated in fibrinolysis (see review by Kluft et al. [4]). This so-called 'intrinsic pathway' of fibrinolysis, to which pro-UK also belongs [5,6], remains poorly characterized and no mechanism by which it targets fibrin clots has been identified.

Although exogenous pro-UK induces fibrin-specific clot lysis in plasma *in vitro* and *in vivo* [1], the biological role of endogenous pro-UK is believed to be restricted to tissue remodeling, inflammation and cell migration [7–9] rather than fibrinolysis. Studies with inhibiting antibodies to urokinase-type plasminogen activator (u-PA) have failed to show any effect on spontaneous lysis of fibrin clots in plasma, which was found to be due exclusively to tissue plasminogen activator (t-PA) [10,11]. Furthermore, the normal plasma concentration

of pro-UK is less than 2 ng/ml [12] and it does not bind to fibrin [13].

These studies of pro-UK-mediated fibrinolysis, and most others in the literature, were conducted in platelet-free systems. By contrast, when clots were made up from platelet-rich instead of platelet-poor plasma, a significant fibrinolytic effect by endogenous pro-UK was identified [11]. We have also previously shown that platelet-rich clots were lysed almost twice as rapidly by exogenous pro-UK as platelet-poor clots, whereas the reverse was the case with t-PA under these conditions [14]. The initial purpose of the present study was to investigate the interaction of pro-UK and platelets in order to help explain these observations. The findings indicated that PK/kallikrein binds to platelets providing a mechanism for the promotion of pro-UK activation by platelet-rich clots. Additionally, it was shown that a portion of pro-UK in blood is found in platelets and that platelets take up exogenous pro-UK from the ambient fluid.

### 2. MATERIALS AND METHODS

Native pro-UK purified from the culture medium of a human kidney tumor cell was obtained from Collaborative Research Inc. (Bedford, MA). Recombinant pro-UK from *E. coli* was obtained from Farnitaha Carlo Erba (Milan, Italy). Low molecular weight (LMW) UK was obtained from Abbott Laboratories (Chicago, IL). Plasma prekallikrein (PK) was obtained from Enzyme Research Laboratory (South Bend, IN) and a polyclonal antibody to PK was obtained from Calbiochem (San Diego, CA). Plasma PK activator (PPA), containing factor XII, ellagic acid, cephalin and HMW kininogen, was obtained from Kabi Pharmacia Hepar (Franklin, OH). Bdekin was purchased from Biopharm (Charleston, SC) and soybean trypsin inhibitor from Sigma (St. Louis, MO).

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*Abbreviations:* Pro-UK, pro-urokinase; UK, urokinase; u-PA, urokinase plasminogen activator; PK, prekallikrein; t-PA, tissue plasminogen activator; PGE, prostaglandin E<sub>1</sub>; PRP, platelet-rich plasma; PPP, platelet-poor plasma; HTA, HEPES Tyrodes-Albumin; HMW, high molecular weight; LMW, low molecular weight; SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; PPA, plasma prekallikrein activator

Human platelets were prepared from venous blood collected into 3.8% citrate (9:1). Platelet-rich plasma (PRP) was obtained by centrifugation at  $160 \times g$  for 15 min at room temperature. After the addition of prostaglandin  $E_1$  ( $PGE_1$ ) ( $1 \mu M$ ), the PRP was centrifuged at  $725 \times g$  and the platelet-poor plasma (PPP) removed. The platelets were resuspended by gentle mixing in HEPES Tyrodes-albumin buffer (HTA) containing 128 mM NaCl, 8.9 mM  $NaHCO_3$ , 5.6 mM dextrose, 10 mM HEPES, 0.35 mg/ml BSA, 12 mM KCl, 3 mM  $KH_2PO_4$ , 3 mM  $MgCl_2$ , pH 7.5. The washing was repeated twice and the supernatant removed after centrifugation each time. Platelet counts were determined in a Coulter counter (Coulter Electronics, Hialeah, FL).

Acid washing of platelets was carried out as follows: A pellet of  $\approx 1 \times 10^8$  washed platelets was resuspended in 1.0 ml of 20 mM citrate (pH 4.5, 0.15 M NaCl, 0.3 mg/ml BSA. After 5 min, the platelets were spun down and resuspended in 10 ml of HTA buffer. After 5 min, the platelet pellet was recovered by centrifugation and then analyzed by zymography. Acid washing by this procedure was shown to completely remove platelet-bound PK and t-PA.

More than 20 preparations from 10 different donors were analyzed.

#### 2.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and casein autography (zymography)

Samples were electrophoresed at 15 mA constant current in 7.5% polyacrylamide slab gels using the buffer system of Laemmli (15). Zymographic analysis was performed according to the method of Granelli-Piperno and Reich [16] modified by Vassalli et al. [17]. After electrophoresis, the acrylamide slabs were washed by agitation for 2 h in 2.5% Triton X-100 in water, followed by 1 h in 0.1 M Tris-HCl (pH 8.0), layered over an underlay consisting of 0.8% agarose (Agarose low melting, Fisher Biotech), casein (2% w/v; Carnation Non-fat Dry Milk) and plasminogen (20–40  $\mu g/ml$ ) in 0.1 M Tris-HCl (pH 8.0) and incubated at  $37^\circ C$  for  $\approx 20$  h.

For most studies of PK, plasminogen-free plates were used. This made it possible to incubate ( $37^\circ C$ ) the casein plate for at least four days, required to visualize PK, without degradation of the casein. Since PK comigrates with plasminogen in SDS-PAGE, we found that it is readily visualized on a plasminogen-free plate by adding plasminogen (4  $\mu g/ml$ ) directly to the sample.

**Sample preparations.** The washed platelet pellet was dissolved in SDS sample buffer (50  $\mu l$ ) for electrophoresis. To determine if activities in the preparations were associated specifically with the platelets, 20  $\mu l$  of the final wash was analyzed for comparison with the platelet pellet which was  $< 4 \mu l$  in volume. All samples were boiled in SDS for 1 min.

#### 2.2. Platelet-associated kallikrein activity and pro-UK activating activity

**Amidolytic activity.** Washed platelets in HTA buffer were incubated with 8  $\mu l/ml$  of PPA for 30 min to activate PK. The assay mixture (100  $\mu l$ ) contained platelets ( $1 \times 10^8/ml$ ) and kallikrein synthetic substrate, S-2302 (0.4 mM) in HTA buffer. The conversion of S-2302 was determined using a microtiter plate reader (Dynatech MR 5000, Dynatech Laboratories, Inc., Chantilly, VA). From the absorbance change at 410/490 nm, the concentration of kallikrein present in the sample was calculated using a calibration curve made under the same conditions with known concentrations of kallikrein. The concentration of active platelet-bound kallikrein was measured as the soybean trypsin-inhibitable (SBTI) activity against S-2302 in separate incubations.

**Pro-UK activating activity.** Activation mixtures (100  $\mu l$ ) were made directly in the microtiter plate and contained  $1 \times 10^8$  platelets/ml in HTA buffer with a range of concentration of pro-UK (20–800 nM) in the presence of 0.6 mM substrate for urokinase (S-2444) at room temperature. The generation of S-2444 activity before and after the addition of PPA to activate PK was measured continuously at 410 nm (against a reference wavelength of 490 nm) on a microtiter plate reader. The effects of PPA and pro-UK alone or in combination on the substrate were tested.

#### 2.3. Incubation of platelets with pro-UK, UK or t-PA

In order to determine if pro-UK was taken up by platelets, washed platelets were incubated ( $37^\circ C$ ) in PPP or HTA buffer ( $\approx 4 \times 10^8$  platelets/ml) enriched with 0.5  $\mu g/ml$  of native or *rec*-pro-UK, HMW-UK, LMW-UK, or t-PA for 5 min. The two UK preparations and t-PA were incubated only in buffer to avoid complexation with inhibitors. Thereafter, the platelets were recovered by centrifugation ( $725 \times g$  for 15 min), washed two times in HTA buffer and then resuspended in bank plasma and reincubated for 0.5, 1, 2 and 22 h. At the end of the final incubation period, the platelets were recovered by centrifugation, washed two times in HTA buffer and examined by zymography on plasminogen enriched casein plates. The corresponding PPP (20  $\mu l$ ) at the end of each incubation period was examined alongside the platelets.

### 3. RESULTS

#### 3.1. The endogenous u-PA intrinsic to platelets and plasma (Fig. 1)

A representative zymogram of platelets ( $1 \times 10^8$ ) washed only once showed a dominant plasminogen activator band of activity at  $\approx 53$  kDa corresponding to u-PA. In addition, a band at  $\approx 70$  kDa could be seen consistent with t-PA, which has previously been shown to bind to platelets by a specific, low affinity binding site [18]. In addition, a  $\approx 100$  kDa band was seen in the platelets, consistent with a UK:plasminogen activator inhibitor-1 (PAI-1) complex (lane 1). The corresponding PPP also showed a band of u-PA activity and a  $\approx 120$  kDa band consistent with a t-PA:PAI-1 complex. The UK:PAI-1 complex was invariably absent in the PPP (lane 2). From other zymograms (not shown) of

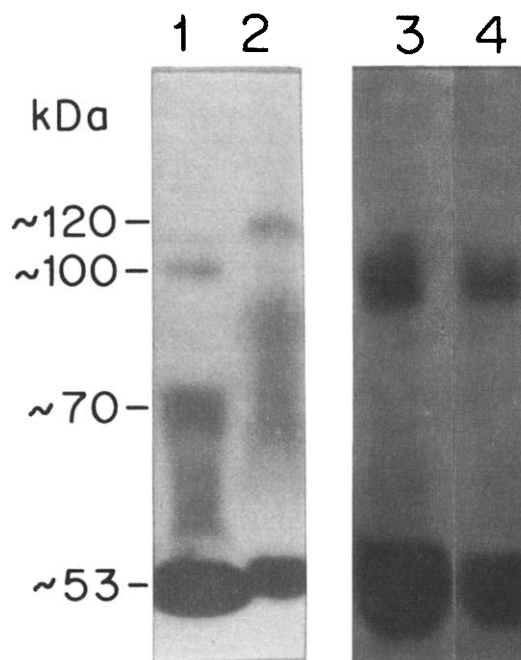


Fig. 1. Zymography (plasminogen-enriched casein) of washed platelets ( $1 \times 10^8$ ) and PPP (20  $\mu l$ ). Lane 1: platelets washed once in HTA buffer; 2: corresponding PPP; 3: platelets preincubated with native pro-UK (0.5  $\mu g/ml$ ) in PPP for 5 min before washing twice in HTA buffer; 4: the same platelets after acid washing.

PPP (1–20  $\mu$ l) and platelets ( $10^7$ – $10^8$ ), it was estimated that  $\approx$  20% of the u-PA in blood was present in platelets based on their zymographic activities. The final wash (20  $\mu$ l HTA buffer) was invariably devoid of activity, indicating that the buffer remaining with the platelet pellet was not the source of the activities seen.

When platelets were washed twice, the t-PA band could no longer be seen (lane 3). Subsequent acid washing of the platelets induced only slight reduction in the intensity of the u-PA or u-PA:PAI-1 complex (lane 4). The increased density of the bands in lanes 3 and 4 was due to additional pro-UK in the platelets as a result of preincubation (5 min at 37°C) in PPP enriched with pro-UK (0.5  $\mu$ g/ml).

### 3.2. Platelet-associated PK and PK in plasma (Fig. 2)

A Comassie stain of prekallikrein and plasminogen showed that they migrated in similar positions on SDS-PAGE (A). This made it possible to add plasminogen to the PK-containing sample and perform zymography on a plasminogen-free plate to overcome the problem of casein disintegration by plasmin over the lengthy period of incubation needed to visualize the PK.

On a plasminogen-free casein plate (incubated 4 days), a band consistent with PK was the only activator visualized in plasma under these conditions. Prekallikrein (50  $\mu$ g/ml) in the absence of plasminogen in the loaded sample induced no lysis (not shown). Plasminogen (4  $\mu$ g/ml) alone induced essentially no lysis. However, PK together with plasminogen in the sample induced a lysis band comparable in position to the single lysis band seen in plasma (B). Similarly, platelets alone induced no lysis on the plasminogen-free plate whereas platelets plus plasminogen induced a lysis band comparable to that induced by plasma and by PK plus plasminogen (C).

The immunoreactivity of the  $\approx$  90 kDa lysis band in plasma and platelets was determined by showing that

when PK antibody was included in the sample, lysis was inhibited. This was also previously observed by Tsuda et al. [6] for the band in this position in normal plasma. The platelet-associated activity was similarly inhibited.

### 3.3. Platelet kallikrein and pro-UK activating activities

Further confirmation that this activity was PK was obtained by activating the platelet preparations with PPA in the presence of kallikrein substrate (S-2302). An activity corresponding to 40–200 pM kallikrein per  $1 \times 10^8$  platelets was found. In the absence of platelets or PPA, no significant substrate activation was induced. No amidolytic activity against UK substrate (S-2444) was detectable in these preparations.

Pro-UK activating activity was assayed with a reaction mixture of platelets, pro-UK (400 nM) and UK synthetic substrate (S-2444) activated with PPA. Urokinase generation occurred as measured by activation of the substrate, which could be inhibited by the addition of soybean trypsin inhibitor to inhibit kallikrein, but not by Bdekin (a specific inhibitor of plasmin). In the absence of platelets, no significant activation of the substrate occurred under these conditions.

### 3.4. Uptake of pro-UK and rec-pro-UK by platelets

When platelets were incubated for 5 min in plasma enriched with 0.5  $\mu$ g/ml pro-UK, there was significant uptake of pro-UK by the platelets as indicated by zymography. This pro-UK was not significantly dissociated by acid washing as previously shown (Fig. 1).

The uptake of pro-UK from the ambient fluid was most apparent when *rec*-pro-UK was used, since due to the absence of carbohydrate, it was clearly resolved migrating ahead of the endogenous pro-UK (Fig. 3). The control platelet preparation is shown (lane 2) alongside the PPP (lane 3). The latter induced no lysis bands due to the relatively short (14 h) incubation time of the plate. The higher MW lysis zone from the en-

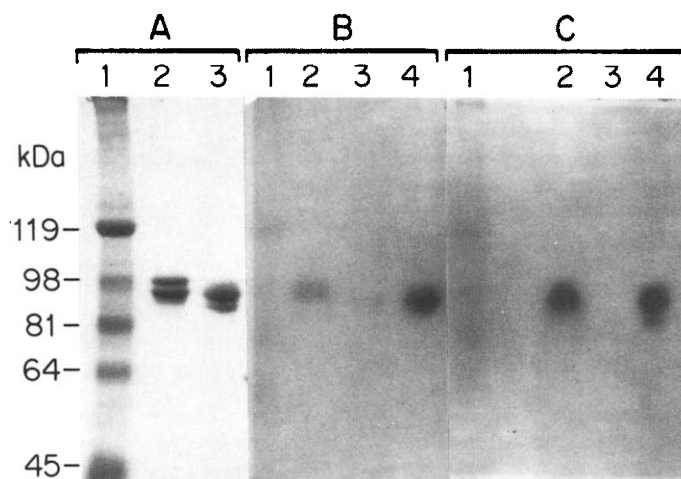


Fig. 2. SDS-PAGE Comassie stained (A) and zymograms on plasminogen-free casein (B and C). A1: MW standards; A2: PK; A3: plasminogen. B1: MW standards; B2: PPP (20  $\mu$ l); B3: plasminogen (4  $\mu$ g/ml); B4: PK (50  $\mu$ g/ml) + plasminogen. C1: MW standards; C2: PK + plasminogen; C3: platelets ( $1 \times 10^8$ ); C4: platelets + plasminogen.

ogenous pro-UK intrinsic to platelets was similar in all the lanes indicating no significant differences in the number of platelets in each sample and that the endogenous pro-UK in platelets was not dissociated by the incubations in PPP for up to 22 h. The platelets incubated with *rec*-pro-UK and then washed and reincubated in normal, unenriched PPP for 30 min (lane 4), 1 h (lane 5), 2 h (lane 6) and 22 h (lane 9) induced comparable lysis zones, indicating no apparent dissociation of the *rec*-pro-UK over this period of time. A small but unchanging amount of *rec*-u-PA activity was seen in the corresponding PPP at the end of each incubation period (lanes 6, 8 and 10) indicating that a fraction of the *rec*-pro-UK was released within the first 30 min, probably representing the unincorporated portion remaining on the receptor. However, the bulk of the platelet-associated *rec*-pro-UK remained unaffected by the incubation, consistent with it having been incorporated. Moreover, this portion of the *rec*-pro-UK also resisted dissociation by acid washing as shown for native pro-UK in Fig. 1. A very faint lysis zone migrating at  $\approx 100$  kDa, consistent with a UK:PAI-1 complex was seen in the platelet preparations (lanes 2, 4, 5, 7 and 9) but not in the PPP suggesting that this complex was also inside the platelets (Fig. 3).

Incubation of platelets with native HMW-UK in HTA buffer induced similar uptake of the UK. Uptake of UK was not accompanied by any more inhibitor complex formation than that seen with pro-UK, suggesting that storage of u-PA in platelets is separate from that of PAI-1 or that most of the latter is in a latent form. By contrast, incubation with LMW-UK or t-PA in HTA buffer at the same concentrations induced no uptake by platelets (data not shown), indicating that the specificity of uptake was comparable to that of the platelet u-PA receptor described by Vaughan et al. [19].

#### 4. DISCUSSION

The present study showing that PK/kallikrein is associated with platelets is based on molecular weight, characteristic response when loaded with plasminogen on a plasminogen-free casein plate, immuno and substrate reactivities and pro-UK activating activity. Since pro-UK activation is importantly involved in its fibrinolytic effect, the findings may explain the observation that platelets promote fibrinolysis by both endogenous [11] and exogenous [14] pro-UK.

The finding that pro-UK is also associated with platelets, localizes two proenzymes of the intrinsic fibrinolytic pathway to a thrombus, where factor XIIa can initiate a sequence of reactions resulting in focal plasmin generation by directly activating PK to kallikrein which in turn activates pro-UK to UK. A relationship between factor XII and fibrinolysis has been suspected for more than 30 years since the observations of Niewiarowski and Prou-Wartelle [20] and was recently further

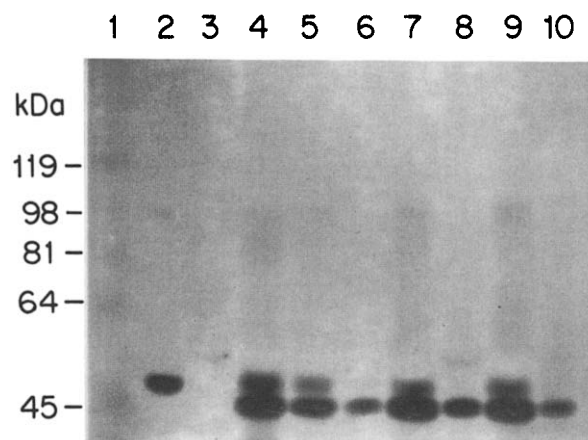


Fig. 3. Zymogram (plasminogen-enriched casein) of washed platelets ( $1 \times 10^8$ ) (lane 1); PPP (2); platelets preincubated ( $37^\circ\text{C}$  for 5 min) in HTA buffer enriched with *rec*-pro-UK ( $0.5 \mu\text{g/ml}$ ), then washed and incubated in normal PPP for 30 min [4]; 1 h [5]; 2 h [7] and 22 h [9]. The corresponding PPP's at the end of each incubation period are shown in lanes 6, 8 and 10.

characterized by Binnema et al. [21]. The extensive laboratory data in the literature on this fibrinolytic pathway is supported by the clinical observations that factor XII deficient patients, notably the index case John Hageman [22], rather than having a bleeding diathesis appeared to be predisposed to both venous thromboembolism [23] and acute coronary thrombosis [24]. However, a mechanism by which the contact dependent intrinsic pathway mediates fibrinolysis has not previously been proposed.

The present finding of platelet-associated pro-UK extends two previous observations. Park et al. found pro-UK associated with sonicated platelet membrane and concluded that it was located in the outer leaflet but no role for it was defined [25]. Vaughan et al. identified the presence of a u-PA receptor on platelets which was, however, of low affinity ( $\approx 130$  nM) and therefore considered to be of uncertain physiological relevance [19]. The specificity of this receptor, which did not bind t-PA or LMW-UK, was similar to the specificity of platelet uptake observed in the present study. Both endogenous pro-UK, intrinsic to platelets, and exogenous pro-UK were incorporated by platelets, as evidenced by a resistance to dissociation by acid washing or by prolonged (22 h) incubation in a pro-UK-poor environment. Since neither LMW-UK nor t-PA were similarly incorporated, it is postulated that the platelet u-PA receptor identified by Vaughan et al. [19] may serve the function of endocytosis. However, studies to determine the location of the pro-UK in platelets remain to be done. It was estimated that platelets contain about 20% of the pro-UK present in the blood of healthy subjects.

The incorporation of exogenous native pro-UK and non-glycosylated *rec*-pro-UK from the ambient plasma into platelets may explain certain intriguing clinical

findings. For example, it has been reported in patients with acute coronary thrombosis, that pro-UK administration is associated with an exceptionally low reocclusion rate [26], which in one study was under 2% [27] as compared with about 20% for t-PA [28] and about 10% for streptokinase [29]. This low reocclusion rate suggests that pro-UK may have a prolonged post-infusion effect which cannot be explained by its very short ( $\approx 7$  min) plasma half-life. Additionally, it has been reported that administration of pro-UK as a single intravenous bolus injection in dogs, an animal in which the  $t_{1/2}$  of pro-UK is equally short [1], induced an unexpected prolonged ( $> 40$  min) thrombolytic effect [30]. These observations could be explained by the present study which suggests that a portion of infused pro-UK may be rapidly incorporated by platelets thereby giving it a long half-life and a potentially useful location for a targeted thrombolytic and antithrombotic effect.

In contrast to pro-UK, the platelet-associated PK was dissociated by extensive washing in HTA buffer or by acid washing, consistent with it binding to a surface protein. To our knowledge, PK has not previously been reported to be associated with platelets. However, a specific binding site on intact platelets for high molecular weight kininogen [31], which also protects it from degradation by kallikrein [32], has been identified. Since PK binds tightly to high molecular weight kininogen [33] and is present in plasma at a relatively high concentration ( $\approx 40 \mu\text{g/ml}$ ) [33], the present finding of platelet-associated PK is not surprising. The postulated significance to intrinsic fibrinolysis of this co-localization of PK and pro-UK by platelets is that it provides a mechanism by which factor XII may trigger thrombus-dependent and targeted plasminogen activation. To date, almost all studies of this pathway have been confined to platelet-free systems which may explain why it has defied delineation.

In conclusion, evidence is presented which indicates that a portion ( $\approx 20\%$ ) of the pro-UK intrinsic to blood is found inside platelets, and that exogenous pro-UK in plasma is rapidly incorporated by platelets. Since t-PA and LMW-UK were not incorporated, this profile is consistent with a previously identified platelet u-PA receptor [19], which is, therefore, implicated in endocytosis. Prekallikrein was found associated with the surface of intact platelets. This co-localization of these two zymogens prompts the postulation that the contact dependent, intrinsic pathway of fibrinolysis is mediated by platelets at the site of a forming thrombus where factor XIIa can initiate the sequence of reactions leading to plasmin generation. In addition, the rapid incorporation of exogenous pro-UK from plasma into platelets may be of significance to the use of pro-UK in therapeutic thrombolysis.

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